Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/011046

International filing date: 01 April 2005 (01.04.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/625,479
Filing date: 04 November 2004 (04.11.2004)

Date of receipt at the International Bureau: 06 May 2005 (06.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

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April 26, 2005

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APPLICATION NUMBER: *60/625,479*
FILING DATE: *November 04, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/11046*

Certified by

Jon W. Dudas

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15866 U.S. PTO 110404

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 367469814 US

17513 U.S. PTO 60/625479 110404

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Mark J.	Cantwell	San Diego, CA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHODS OF USING 5,10-METHYLENE HYDROFOLATE TO TREAT CANCER

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 33 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 21 ☐ Other (specify)

☐ Application Date Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

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Respectfully submitted,

SIGNATURE

Date November 4, 2004

REGISTRATION NO. 38,710 *(if appropriate)*

Docket Number: ADX-00107.P.1

TYPED or PRINTED NAME David R Preston

TELEPHONE 858-724-0375 x102

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Docket Number ADX-00107.P.1

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Joan M.	Robbins	San Diego CA

Number 1 of 1

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FEE TRANSMITTAL for FY 2003

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☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	To be determined
Filing Date	Herewith
First Named Inventor	CANTWELL
Examiner Name	To be determined
Art Unit	To be determined
Attorney Docket No.	ADX-00107.P.1

METHOD OF PAYMENT *(check all that apply)*

☑ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims		-20** =		X =
Independent Claims		- 3** =		X =
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBTOTAL (3) ($) 0.00

SUBMITTED BY				(Complete *(if applicable)*)
Name (Print/Type)	David R. Preston	*Registration No. (Attorney/Agent)*	38,710	*Telephone* 858-724-0375
Signature				*Date* Nov 4, 2004

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Mail Stop Provisional Application
"Express Mail" Mailing Label Number: EV 367469814 US

Date of Deposit: November 4, 2004

Commissioner for Patents
Alexandria, VA 22313-1450

Re: Provisional Patent Application
Entitled: METHODS OF USING 5,10-METHYLENE HYDROFOLATE TO TREAT CANCER
Appl. No.: To be determined
Filed: Herewith
Inventor: CANTWELL, Mark; ROBBINS, Joan
Our Ref.: ADX-00107.P.1

Sir:

The following documents are forwarded herewith for appropriate action by the United States Patent and Trademark Office:

1. Provisional Application for Patent Cover Sheet (in duplicate);

2. Fee transmittal (in duplicate);

3. Complete U.S. Provisional Patent Application entitled:

METHODS OF USING 5,10-METHYLENE HYDROFOLATE TO TREAT CANCER

and naming as inventors

CANTWELL, Mark; ROBBINS, Joan

the provisional application comprising:

Total pages of application: [55];
Pages of specification: [33];
Sheets of Figures: [21]; and
Pages of Title Page: [1].

4. One Return Post Card; and

5. Our Check for $80.00 to cover the Application Fee.

It is respectfully requested that the attached postcard be stamped with the filing date and unofficial application number and returned as soon as possible.

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The following attorney and agent are the attorney and agent of record for prosecuting this application and transacting all business in the USPTO connected therewith:

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Respectfully Submitted,
DAVID R. PRESTON & ASSOCIATES, A.P.C.

David R. Preston
Attorney for Applicant
Registration No. 38,710

PROVISIONAL

PATENT APPLICATION

on

METHODS OF USING 5,10-METHYLENE HYDROFOLATE TO TREAT CANCER

by

Mark J. Cantwell and Joan M. Robbins

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David R. Preston & Associates
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ADX-00107.P.1

METHODS OF USING 5,10-METHYLENE TETRAHYDROFOLATE TO TREAT CANCER

Cancer is a major public health concern. Colorectal cancer alone cases approximately 50,000 deaths per year in the United States. Nearly half of the approximately 130,000 cases of colorectal cancer that are diagnosed every year present with or develop into metastatic disease, for which chemotherapy is the only treatment. New effective drug-based therapies for treatment are urgently sought not only for colorectal cancers, but for other cancers such as but not limited to breast cancer, pancreatic cancer, stomach cancers, hepatic cancer, bladder cancer, cervical cancer, head and neck cancer, lung cancer, ovarian cancer, and prostate cancer. The present invention provides new drug-based methods of cancer treatment, including methods that can provide reduced toxicity to the patient and greater efficacy than current modalities.

The anticancer drug 5-fluorouracil (5-FU) is an inhibitor of thymidylate synthase (TS), an enzyme required for nucleic acid biosynthesis. 5-FU used to treat cancers such as colorectal and breast cancer, is commonly used in conjunction with folinic acid (leucovorin), which is converted intracellularly into reduced folate, a cofactor for TS. Toxicities associated with 5-fluorouracil include stomatitis, mucositis, gastrointestinal symptoms, and hematological toxicity, particulary neutropenia, thrombocytopenia, and leucopenia.

There is a need to develop improved anti-cancer drug regimens that increase survivorship with reduced toxicity. Clinical trials have demonstrated that administration of 5,10-methylene tetrahydrofolate, a form of reduced folate used as a cofactor by TS, along with 5-FU, increases the length or remissions in patients with breast and gastrointestinal cancer when compared with the use of folinic acid (leucovorin) combined with 5-FU.

Detailed Description of the Invention

The present invention is based on the surprising result that 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA), while increasing the efficacy of 5-fluoruracil (5-FU) in reducing the rate of tumor growth and increasing survivorship, also reduces the toxicity to the patient of 5-FU. As disclosed herein, treatment with 5,10-CH_2-THFA and 5-FU reduces tumor growth rate and increases survivorship of tumor-bearing animals with respect to treatment with either 5-FU alone, or 5-FU in combination with folinic acid (FA; leucovorin), while demonstrating less toxicity than either treatment.

The present invention is further based on the finding that treatment of tumor-bearing animals with 5,10-CH_2-THFA and 5-FU and additional anticancer drugs can also improve outcomes with respect to single modality treatments or alternative combination treatments that include the use of 5-FU with folinic acid (leucovorin).

The present invention provides:

1. Methods for decreasing the toxicity to a patient of a cancer drug treatment regimen that includes administration of 5-fluorouracil (5-FU) to a cancer patient by co-administering 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA). The methods include treatments in which the toxicity of treatment with 5-FU is reduced by administering 5,10-CH_2-THFA instead of folinic acid as a source of TS cofactor.

2. Methods for decreasing mortality caused by toxicity of chemotherapeutic agents. In one aspect, the present invention includes methods for decreasing mortality caused by toxicity of 5-fluorouracil (5-FU) by co-administering 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA. The methods include treatments in which patient mortality is decreased in patients treated with 5-FU by administering 5,10-CH_2-THFA instead of folinic acid as a source of TS cofactor.

3. Methods of treating cancer patients with combination chemotherapy involving 5-fluorouracil (5-FU), 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA), and one or more additional anti-cancer drugs. Treating cancer patients with 5,10-CH_2-THFA, 5-FU, and one or more additional anti-cancer drugs can reduce the rate of tumor growth or increase

the survivorship of cancer patients when compared with treating patients with the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA and 5-FU, or when compared with treating patients with 5-FU and the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA, or when compared with treating patients with 5-FU and folinic acid and the one or more additional anti-cancer drugs.

4. In yet another aspect, the present invention includes methods for decreasing mortality caused by toxicity of treatment of patients with 5-fluorouracil (5-FU) and at least one other chemotherapeutic agent by additionally administering 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA). In some aspects, the present invention includes methods of decreasing mortality of patients treated with with 5-fluorouracil (5-FU) and at least one other chemotherapeutic agent by additionally administering 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA). The methods include treatments in which mortality is decreased in patients treated with 5-FU and an additional chemotherapeutic agent (other than folinic acid) by administering 5,10-CH_2-THFA instead of folinic acid as a source of TS cofactor. Treating cancer patients with 5,10-CH_2-THFA, 5-FU, and one or more additional anti-cancer drugs can decrease mortality when compared with treating patients with 5-FU and the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA, or when compared with treating patients with 5-FU and folinic acid and the one or more additional anti-cancer drugs.

5. The present invention includes methods of increasing the dose of a chemotherapeutic agent. In these aspects, the present invention includes methods of increasing the dose of a chemotherapeutic agent administered in combination therapy with 5-FU by co-administering 5,10-CH_2-THFA. The reduction in toxicity associated with co-administration of 5,10-CH_2-THFA with 5-FU can allow dosages to be used that would be prohibitively toxic when folinic acid is co-administered with 5-FU. These methods include methods of increasing the dose of 5-FU co-administered with 5,10-CH_2-THFA beyond the range typically used for 5-FU when administered with folinic acid. The methods also include methods of increasing the dose of an additional chemotherapeutic agent beyond the range typically used when the additional chemotherapeutic agent is

administered in combination therapy with 5-FU by co-admininstering 5,10-CH_2-THFA. The methods also include methods of increasing the dose of an additional chemotherapeutic agent beyond the range typically used when the additional chemotherapeutic agent is administered in combination therapy with 5-FU by co-admininstering 5,10-CH_2-THFA in place of folinic acid.

I. METHODS FOR DECREASING THE TOXICITY TO A PATIENT OF A CANCER DRUG TREATMENT REGIMEN THAT INCLUDES ADMINISTRATION OF 5-FLUOROURACIL (5-FU) BY CO-ADMINISTERING 5,10-METHYLENE TETRAHYDROFOLATE (5,10-CH_2-THFA)

One aspect of the present invention is methods for decreasing the toxicity of a cancer drug treatment that includes administration of 5-fluorouracil (5-FU). The method comprises administering 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA) to the patient before, after, or concurrent with the administration of 5-FU to reduce the toxicity of 5-FU. In preferred embodiments of this aspect of the present invention, 5-FU and 5,10-CH_2-THFA are administered to the patient in the absence of folinic acid (FA; leucovorin). In some preferred embodiments of this aspect of the present invention, 5,10-CH_2-THFA is administered to a patient receiving 5-FU to reduce hematological toxicity of 5-FU. In some preferred embodiments of this aspect of the present invention, 5,10-CH_2-THFA is administered to a patient receiving 5-FU and a TS cofactor or cofactor precursor, where 5,10-CH_2-THFA is administered instead of folinic acid (FA, leucovorin), to prevent the hematological toxicity associated with treatment with 5-FU and a TS cofactor (or cofactor precursor).

The invention is based on the surprising result that 5,10-methylene tetrahydrofolate, while increasing the efficacy of 5-FU in reducing the rate of tumor growth and increasing survivorship, also reduces the toxicity of 5-FU towards nontumor cells. As disclosed in Examples 1 and 2, treatment with 5,10-CH_2-THFA and 5-FU reduces tumor growth rate and increases survivorship of tumor-bearing animals with respect to treatment with either 5-FU alone, or 5-FU in combination with folinic acid (leucovorin), while demonstrating less toxicity to the animal than either treatment.

As used herein, "reduce the toxicity" refers to reducing toxic systemic effects on the patient, or toxic effects on the noncancerous cells of the patient. Toxicity can include,

as nonlimiting examples, increased lacrimation; mucositis; esophagopharyngitis; neurological toxicity, such as parasthesias, insomnia, and dizziness; gastrointestinal toxicity, such as nausea, vomiting, and diarrhea; weight loss toxicity; cardiac toxicity; dermatological toxicity, including alopecia, sweating, and rashes; and hematological toxicity, such as, but not limited to, neutropenia, thrombocytopenia, lymphopenia, and leucopenia.

In preferred embodiments of this aspect of the present invention, 5,10-CH_2-THFA is administered along with 5-FU to reduce the degree of hematological toxicity associated with 5-FU treatment. For example, administering 5,10-CH_2-THFA along with 5-FU can reduce neutropenia, thrombocytopenia, lymphopenia, or leucopenia associated with chemotherapy regimens that include 5-FU, including but not limited to chemotherapy regimens that include 5-FU and folinic acid (leucovorin).

A cancer patient can be a patient with any type of cancer. In some preferred embodiments of the present invention in which 5,10-CH_2-THFA is administered to a cancer patient receiving 5-FU, the patient has a tumor type that is currently treated with 5-FU, such as, for example, colorectal carcinoma, pancreatic, breast, or stomach cancer.

Those skilled in the art of cancer treatment and chemotherapy would be able to determine optimal dosages and regimens for 5,10-CH_2-THFA and 5-FU. Some preferred treatments of cancer patients with 5-FU and 5,10-CH_2-THFA are regimens using from 10 milligrams to 1 gram of 5,10-CH_2-THFA per m^2, preferably from 25 milligrams to 500 milligrams of 5,10-CH_2-THFA per m^2, and more preferably from about 50 milligrams to about 250 milligrams of 5,10-CH_2-THFA per m^2. For example, a preferred dose of 5,10-CH_2-THFA can be from about 100 to about 200 milligrams per m^2. Dosage of 5-FU can be from about to about 25 milligrams to about 5 grams per m^2, and is preferably from about 50 milligrams to 2.5 grams per m^2, and more preferably from about 100 milligrams to about 1 gram per m^2. For example, a preferred dose of 5-FU can be from about 250 to about 700 milligrams per m^2.

The drugs can be administered intravenously or by any other feasible means, according to regimens that can be determined by qualified clinicians. For example, bolus injection of each drug can be given once weekly for a number of weeks. Preferably, 5,10-CH_2-THFA is administered prior to 5-FU. For example, the patient can receive the 5,10-

CH_2-THFA dose from about 10 minutes to about four hours prior to receiving the 5-FU dose. We also propose 5,10-CH_2-THFA use with new formulations of 5-FU, specifically oral forms of 5-FU (e.g. Xeloda, capecitabine).

II. METHODS OF TREATING CANCER PATIENTS WITH COMBINATION CHEMOTHERAPY INVOLVING 5-FLUOROURACIL (5-FU), 5,10-METHYLENE TETRAHYDROFOLATE (5,10-CH_2-THFA), AND ONE OR MORE ADDITIONAL ANTI-CANCER DRUGS.

One aspect of the present invention is methods for treating cancer patients with combination chemotherapy that includes administration of 5-fluorouracil (5-FU), 5,10-CH_2-THFA, and one or more additional anti-cancer drugs. The method comprises administering 5-FU, 5,10-CH_2-THFA, and one or more additional drugs to a cancer patient in the absence of folinic acid (leucovorin). As used herein, an "additional" anti-cancer drug is an anti-cancer drug that is not 5,10-methylene tetrahydrofolate (5,10-CH_2-THFA), 5-fluorouracil (5-FU), or folinic acid (FA; leucovorin).

An anti-cancer drug can be any drug used to treat cancer, including small molecules, large molecules, peptides, nucleic acids and nucleic acid analogues (such as, but not limited to antisense molecules, ribozymes, and siRNAs), and antibodies or antibody fragments. As nonlimiting examples, anticancer drugs used in combination therapy with 5-FU and 5,10-CH_2-THFA can be topoisomerase inhibitors (e.g., irinotecan), antimetabolite drugs (e.g., methotrexate, gemcitabine), alkylating agents (e.g., cyclophosphamide), nucleic acid biosynthesis inhibitors (e.g., mitomycin, doxorubicin, cisplatin, oxaliplatin), microtubule disrupting drugs (e.g., paclitaxel, vincristine), hormone blocking drugs (e.g., tamoxifen), inhibitors of kinases, including but not limited to receptor and nonreceptor tyrosine kinases (e.g., Iressa, Tarceva, SU5416, PTK787, Gleevec), proteosome inhibitors (e.g., bortezomib), immune modulators (e.g., levamisole), cytokines (e.g., interleukins, tumor necrosis factors) and drugs that inhibit the activity of cytokines, hormones, or receptors for cytokines or hormones (e.g., bevacizumab, avastin). An anti-cancer drug can also be a drug under investigation for potential anti-cancer activity, such as those listed in **Table 1**. Anti-cancer drugs include monoclonal antibodies, such as but not limited to monoclonal

antibodies that bind cytokines, hormones, or hormone receptors (e.g., antibodies that block activation of EGF or VEGF growth factors, such as Avastin, erbutux, herceptin), etc.

A cancer patient can be a patient with any type of cancer. In some preferred embodiments of the present invention in which 5,10-CH_2-THFA is administered to a cancer patient receiving 5-FU, the patient has a tumor type that is currently treated with 5-FU, such as, for example, colorectal carcinoma, pancreatic, breast, or stomach cancer. The inventors also contemplate that combination therapies that use 5,10-CH_2-THFA, 5-FU, and one or more additional anti-cancer drugs have potential for treating cancers other than those currently commonly treated with 5-FU.

In some embodiments of this aspect of the present invention, treating a cancer patient with 5,10-CH_2-THFA, 5-FU, and one or more additional anti-cancer drugs can reduce the rate of tumor growth in a cancer patient when compared with treating the patient with the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA and 5-FU, or when compared with treating a patient with 5-FU and the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA.

In some embodiments of this aspect of the present invention, treating cancer patients with 5,10-CH_2-THFA, 5-FU, and one or more additional anti-cancer drugs can increase the survivorship of cancer patients when compared with treating cancer patients with the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA and 5-FU, or when compared with treating cancer patients with 5-FU and the one or more additional anti-cancer drugs in the absence of 5,10-CH_2-THFA.

In some embodiments of this aspect of the present invention, addition of 5,10-CH_2-THFA to a treatment regimen that includes 5-FU and an additional anti-cancer drug can reduce the toxicity to the patient of treatment with 5-FU and one or more additional anti-cancer drugs. As used herein, "reduce the toxicity" refers to reducing toxic systemic effects on the patient, or toxic effects on the noncancerous cells of the patient. Toxicity can include, as nonlimiting examples, increased lacrimation; mucositis; esophagopharyngitis; neurological toxicity, such as parasthesias, insomnia, and dizziness; gastrointestinal toxicity, such as nausea, vomiting, and diarrhea; weight loss toxicity; cardiac toxicity; dermatological toxicity, including alopecia, sweating, and rashes; and

hematological toxicity, such as, but not limited to, neutropenia, thrombocytopenia, lymphopenia, and leucopenia.

Thus, the present invention includes a method of reducing the toxicity to the patient of a drug regimen for cancer treatment that includes 5-FU and one or more additional anti-cancer drugs, comprising adding to the drug regimen 5,10-CH_2-THFA. In some embodiments, the reduced toxicity of 5-FU when combined with 5,10-CH_2-THFA can permit drug regimens in which 5,10-CH_2-THFA and 5-FU are used in combination with the one or more additional anti-cancer drugs that would be prohibitively toxic in the absence of CH_2-THFA.

In embodiments in which addition of 5,10-CH_2-THFA to a treatment regimen that includes 5-FU and an additional anti-cancer drug can reduce the toxicity to a patient of treatment with 5-FU and the additional anti-cancer drug, the inventors contemplate that dosage of at least one of the one or more additional anti-cancer drugs can be administered at an increased dosage relative to the dosage typically used for the one or more additional anti-cancer drugs. Thus, the invention includes a method of increasing the dosage of at least one additional anti-cancer drug used in a drug regimen for treating cancer that includes 5-FU, comprising adding to the drug regimen 5,10-CH_2-THFA.

For example, because of the anti-tumor activity and decreased systemic toxicity of 5,10-CH_2-THFA compared to folinic acid (leucovorin), and because of the similar chemical and metabolic pathways of folinic acid and 5,10-CH_2-THFA, we hypothesize 5,10-CH_2-THFA can substitute for leucovorin in a range of current chemotherapy regiments. Current drugs commonly used in combination with 5-FU plus leucovorin are Irinotecan (CPT-11) and Oxaliplatin. The present invention includes treatments that substitute 5,10-CH_2-THFA for leucovorin in these regiments. Substitution of 5,10-CH_2-THFA for leucovorin can provide equivalent or enhanced therapeutic effects with reduced toxicity. As nonlimiting examples, current drug combination regiments that 5,10-CH_2-THFA can substitute for leucovorin include:

- AIO regimen (folic acid, 5-FU, Irinotecan):
    - Irinotecan (100 mg/m^2) as a 2-hour infusion day 1; leucovorin (500 mg/m^2) as a 2-hour infusion day 1; followed by 5-FU (2,000 mg/m^2)

intravenous (IV) bolus via ambulatory pump over 24 hours weekly x 4 every 52 weeks.

- Douillard regimen (folic acid, 5-FU, Irinotecan):
  - Irinotecan (180 mg/m^2) as a 2-hour infusion day 1; leucovorin (200 mg/m^2) as a 2-hour infusion days 1 and 2; followed by a loading dose of 5-FU (400 mg/m^2) IV bolus, then 5-FU (600 mg/m^2) via ambulatory pump over 22 hours days 1 and 2 every 2 weeks.

- FOLFOX4 regimen (oxaliplatin, leucovorin, 5-FU):
  - Oxaliplatin (85 mg/m^2) as a 2-hour infusion day 1; leucovorin (200 mg/m^2) as a 2-hour infusion days 1 and 2; followed by a loading dose of 5-FU (400 mg/m^2) IV bolus, then 5-FU (600 mg/m^2) via ambulatory pump over 22 hours days 1 and 2 every 2 weeks.

- FOLFOX6 regimen (oxaliplatin, leucovorin, 5-FU):
  - Oxaliplatin (85-100 mg/m^2) as a 2-hour infusion day 1; leucovorin (400 mg/m^2) as a 2-hour infusion day 1; followed by a loading dose of 5-FU (400 mg/m^2) IV bolus on day 1, then 5-FU (2,400-3,000 mg/m^2) via ambulatory pump over 46 hours every 2 weeks.

- FOLFIRI regimen (folic acid, 5-FU, Irinotecan):
  - Irinotecan (180 mg/m^2) as a 2-hour infusion day 1; leucovorin (400 mg/m^2) as a 2-hour infusion day 1; followed by a loading dose of 5-FU (400 mg/m^2) IV bolus on day 1, then 5-FU (2,400-3,000 mg/m^2) via ambulatory pump over 46 hours every 2 weeks.

- IFL (or Saltz) regimen (Irinotecan, 5-FU, leucovorin):
  - Irinotecan (125 mg/m^2), 5-FU (500 mg/m^2) IV bolus, and leucovorin (20 mg/m^2) IV bolus weekly for 4 out of 6 weeks.

The forgoing examples are not intended to be limiting in any way. For example, dosages and regimens can be altered or optimized to minimize toxicity to the patient or improve efficacy. In addition, many anti-cancer drugs that are not described herein can be combined with 5,10-CH_2-THFA and 5-FU. We also propose 5,10-CH_2-THFA use in combination therapies with next-generation forms of 5-FU, specifically oral forms of 5-FU (e.g. Xeloda, capecitabine).

Other uses of 5,10-CH_2-THFA are in combination therapy with new classes of biologic anti-tumor reagents, such as monoclonal antibodies with anti-tumor activity. Examples of antibodies that might be combined with 5,10-CH_2-THFA (preferably with 5-FU) include anti-VEGF antibody (e.g. Avastin, Bevacuzimab) and anti-EGF receptor (e.g. Erbitux, cetuximab, herceptin). As shown in the Examples, combination 5-FU/5,10-CH_2-THFA /Avastin treatment of colorectal carcinoma in nude mice inhibits tumor growth more than the other drug combinations.

Because of the lower toxicity profile of 5,10-CH_2-THFA disclosed herein, the present invention also includes 5,10-CH_2-THFA use in combination with drugs that typically are considered too toxic for widespread use. For example, 5-FU/5,10-CH_2-THFA /Cisplatin therapy is a hypothetical combination. Cisplatin, a platinum-based chemotherapy agent is highly toxic. In addition, the lower toxicity profile of 5,10-CH_2-THFA might allow use of either increased concentrations of drugs (e.g. 5-FU) or prolonged dosing periods. In turn this might improve drug efficacy.

The present invention also includes the use of 5,10-CH_2-THFA in place of folinic acid (leucovorin) in therapies that do not use 5-FU. For example, based on the lower toxicity profile and increased activity of 5,10-CH_2-THFA (CoFactor) compared tofolinic acid(leucovorin), 5,10-CH_2-THFA can be used for methotrexate rescue therapy. This mode of therapy currently uses leucovorin.

EXAMPLE 1: NUDE MOUSE STUDY ON COLORECTAL TUMOR HT-29 TREATMENT WITH 5-FU, 5,10-CH_2-THFA, FA, ANTI-VEGF, AND OXALIPLATIN.

Materials and Methods

Mice

Nude (nu/nu) mice were obtained from Charles River Laboratories. Mice were 6-8 weeks old at the start of all studies. Mice were maintained in isolated, hepa-filter ventilated cages with 4 mice per cage at LAB International's vivarium (San Diego, CA).

Cell Lines

The human colon carcinoma HT-29 was obtained from American Tissue Culture Collection (ATCC). Cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS), 2mM l-glutamine, 100 units/ml penicillin, and 100 micrograms/ml streptomycin (DMEM-10) in a 37°C, 5% CO_2 humidified incubator. Cell lines were passaged every 2-3 days prior to *in vivo* experiments.

Drugs

5-Fluorouracil (5-FU) was obtained from Calbiochem. Folinic acid (leucovorin) and oxaliplatin were obtained from Sigma-Aldrich. CoFactor (5,10 methylenetetrahydofolate) was manufactured by Eprova AG. A monoclonal antibody to vascular endothelial growth factor (anti-VEGF) was either obtained from R&D Systems (clone 26503 recognizing the human VEGF isoform 165) or Genentech (Avastin).

HT-29 Colorectal Carcinoma Nude Mouse Study #1

HT-29 cells were prepared for injection as follows. Confluent tissue culture flasks of HT-29 cells were washed once with PBS followed by cell detachment with trypsin. Detached cells were then washed once in DMEM-10 followed by one wash with PBS. Finally, cells were resuspended at $2x10^7$ cells/ml in PBS. Nude mice (nu/nu) were inoculated subcutaneously with 100 microliters ($2x10^6$ cells) of HT-29 cells using a 28 gauge insulin needle/syringe. When tumors reached 100 to 300 mm^3 in volume, mice were treated with various combinations of 5-FU, CoFactor, leucovorin, oxaliplatin, and anti-VEGF (R&D Systems antibody) administered by intraperitoneal injection. All drugs were dosed daily (0.6 mg/mouse/drug) for five consecutive days with the exception of anti-VEGF and oxaliplatin. Anti-VEGF was dosed once (100 microgram/mouse) on day 5. Oxaliplatin was dosed once on day 1 (0.3mg/mouse). In addition, CoFactor or leucovorin were injected 20 minutes prior to 5-FU injection. Tumor sizes were measured every 2 to 3 days using calipers. Tumor volume was calculated using the following formula: tumor volume = (length x $width^2$)/2. Mice were euthanized by CO_2 followed by cervical dislocation either when a tumor reached >2cm in diameter or upon tumor ulceration.

Data Analysis

Statistical analysis of tumor and blood data was performed using GraphPad Prism scientific software. Bonferonni's T test was used to compare tumor sizes between multiple groups. The Logrank test was used to determine statistical differences between group survival curves. In some cases, in which only two groups were compared, Student's T test was used to determine the significance between group measurements.

Results

Nude mice were treated with the drug combinations described in **Table 2**. In this study, we wanted to examine if combining 5-FU/CoFactor treatment with the oxaliplatin or anti-VEGF antibody (obtained from R&D Systems) could inhibit colorectal tumor growth more than other drug combinations. Drug concentrations and treatment days are described in the materials and methods section. Following treatment, tumor sizes were measured every 2-3 days and tumor volumes calculated. Tumor volumes were then plotted versus time from treatment initiation (**Figures 1** and **2**). To simplify the graphs, we divided analysis into graphs containing anti-VEGF data and another set with oxaliplatin data. Best-fit curves for each treatment group were calculated and plotted in these figures. As seen in **Figure 1**, 5-FU/CoFactor/anti-VEGF treated mice had the slowest tumor growth curve followed by either 5-FU/CoFactor or 5-FU/anti-VEGF treated mice

We also analyzed the differences between mean tumor volumes following treatment. Comparing the various treatment combinations for the anti-VEGF set of data (**Figure 3**), we observed the mean tumor volume of 5-FU/CoFactor/anti-VEGF treated mice (478.6 $\pm$ 102.7, mean $\pm$ SEM, n = 7) was less than 5-FU (752.5 $\pm$ 104.2, n = 8), 5-FU/Leucovorin (707.5 $\pm$ 93.6, n = 8), 5-FU/CoFactor (522.5 $\pm$ 78.2, n = 8), and 5-FU/anti-VEGF (502.5 $\pm$ 64.1, n=8) treated mice. Oxaliplatin treated mice had the largest tumors (tumor volume 875.0 + 90.6, mean + SEM, n =8) (Figure 4), indicating that the HT-29 tumor was not responsive to this drug (see Plasencia et al. (2002) American Society for Clinical Oncology Annual Meeting Abstract No. 2188.) This probably accounts for the lack of equivalent tumor inhibition in the treatment group receiving the triple drug combination of 5-FU/CoFactor/Oxaliplatin (735.0 $\pm$ 80.3, n = 8) (**Figure 4**),

when compared with the triple combination 5-FU/CoFactor/anti-VEGF treated mice, which had the smallest tumor sizes of any anti-VEGF combination (**Figure 3**).

Mouse survival curves were also calculated for all treatment groups. Mice were euthanized upon overt systemic toxicity, tumor ulceration, or when tumor diameter reaches >2cm. At the completion of the study period (42 days), 75% of mice treated with 5-FU/CoFactor were still alive (**Figure 5**). This survival was significantly longer than mice treated with only 5-FU (25%, $p < 0.05$, Logrank test). In addition to 5-FU/CoFactor treated mice, 5-FU/CoFactor/anti-VEGF treated mice also survived longer (57%) than all other treatment groups. The lack of protection of mice treated with 5-FU/CoFactor/Oxaliplatin (25%) **(Figure 6)** compared to other treatment groups can most likely be attributed to the apparent resistance of the HT-29 tumor to oxaliplatin **(Figure 3).** For the oxaliplatin treatment subgroup analysis, 5-FU/CoFactor treatment provided the greatest survival benefit.

EXAMPLE 2: NUDE MOUSE STUDY ON COLORECTAL TUMOR HT-29 TREATMENT WITH 5-FU, 5,10-CH_2-THFA, FA, ANTI-VEGF, AND OXALIPLATIN.

Materials and Methods

Mice

Nude (nu/nu) mice were obtained from Charles River Laboratories. Mice were 6-8 weeks old at the start of all studies. Mice were maintained in isolated, hepa-filter ventilated cages with 4 mice per cage at LAB International's vivarium (San Diego, CA).

Cell Lines

The human colon carcinoma HT-29 was obtained from American Tissue Culture Collection (ATCC). Cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS), 2mM l-glutamine, 100 units/ml penicillin, and 100 micrograms/ml streptomycin (DMEM-10) in a 37°C, 5% CO_2 humidified incubator. Cell lines were passaged every 2-3 days prior to *in vivo* experiments.

Drugs

5-Fluorouracil (5-FU) was obtained from Calbiochem. Folinic acid (leucovorin) and oxaliplatin were obtained from Sigma-Aldrich. CoFactor (5, 10 methylenetetrahydofolate) was manufactured by Eprova AG. A monoclonal antibody to vascular endothelial growth factor (anti-VEGF) was either obtained from R&D Systems (clone 26503 recognizing the human VEGF isoform 165) or Genentech (Avastin).

HT-29 Colorectal Carcinoma Nude Mouse Study #2

HT-29 cells were prepared for injection as follows. Confluent tissue culture flasks of HT-29 cells were washed once with PBS followed by cell detachment with trypsin. Detached cells were then washed once in DMEM-10 followed by one wash with PBS. Finally, cells were resuspended at $1x10^7$ cells/ml in PBS. Nude mice (nu/nu) were inoculated subcutaneously with 100microliters (10^6 cells) of HT-29 cells using a 28 gauge insulin needle/syringe. When tumors reached 30 to 100 mm^3 in volume, mice were treated with various combinations of 5-FU, CoFactor, leucovorin, and anti-VEGF (Genentech's Avastin antibody) administered by intraperitoneal injection. All drugs were dosed daily (0.6 mg/mouse/drug) for seven consecutive days with the exception of anti-VEGF, dosed twice (100 micrograms/mouse) on days 1 and 7. In addition, CoFactor or leucovorin were injected 20 minutes prior to 5-FU injection. Tumor sizes were measured every 2 to 3 days using calipers. Tumor volume was calculated using the following formula: tumor volume = (length x $width^2$)/2. Mice were euthanized by CO_2 followed by cervical dislocation either when a tumor reached >2cm in diameter or upon tumor ulceration.

Data Analysis

Statistical analysis of tumor and blood data was performed using GraphPad Prism scientific software. Bonferonni's T test was used to compare tumor sizes between multiple groups. The Logrank test was used to determine statistical differences between group survival curves. In some cases, in which only two groups were compared, Student's T test was used to determine the significance between group measurements.

Results

Based on the pilot results obtained in the first nude mouse study described above, we repeated another HT-29 nude mouse study with some modifications to study design. Modifications included larger group sizes, substitution of Genentech's anti-VEGF Avastin antibody for R&D System's antibody, exclusion of oxaliplatin, increased number of treatment days, and increased the number of doses of the anti-VEGF antibody. Nude mice were treated with the drug combinations described in **Table 3**. In this study, we wanted to examine if combining 5-FU/CoFactor treatment with the anti-VEGF antibody Avastin could inhibit colorectal tumor growth more than other drug combinations. Drug concentrations and treatment days are described in the materials and methods section. Following treatment, tumor sizes were measured every 2-3 days and tumor volumes calculated. Tumor volumes were then plotted versus time from treatment initiation (**Figure 7**). Best-fit curves for each treatment group were calculated and plotted in this figure. Based on the best-fit curve analysis, the average doubling time for each group was calculated (**Table 4**). Mice treated with the combination of 5-FU/CoFactor/Avastin displayed the slowest growth kinetics (doubling time = 9.9 days) compared to all other groups. These results are consistent with results obtained in the first nude mouse tumor study described earlier.

We also analyzed the differences between mean tumor volumes determined 19 days following treatment initiation. The mean tumor volumes $\pm$ SEM are plotted in figure 8. We observed the mean tumor volume of 5-FU/CoFactor/Avastin treated mice (94.0 ± 10.2, mean $\pm$ SEM, n =12) was significantly less ($p<0.05$, Bonferonni's T test) than 5-FU (368.5 ± 63.7, n = 10), 5-FU/Leucovorin (262.0 ± 36.5, n =11), 5-FU/CoFactor (225.4 ± 32.0, n=12), 5-FU/Avastin (225.5 ± 28.8, n=12), but not 5-FU/Leucovorin/Avastin (140.8 ± 20.3, n=12) treated mice. In contrast, mean tumor volumes of 5-FU/Leucovorin/Avastin treated mice were only significantly smaller than tumor volumes in 5-FU treated mice but not other treatment groups.

Mouse survival curves were also calculated for all treatment groups. Mice were euthanized upon overt systemic toxicity, tumor ulceration, or when tumor diameter reached >2cm. Prior to study completion (38 days from treatment initiation), $\leq$50% of mice treated with saline, 5-FU, or 5-FU plus Avastin were still alive (**Figure 9**). In contrast, 92% of mice treated with 5-FU plus Avastin in combination with either

CoFactor or leucovorin were still alive. This pattern of survival for the various drug combinations is similar to the results observed in the first nude mouse colorectal tumor study described above.

EXAMPLE 3: BLOOD ANALYSIS OF BALB/C MICE TREATED WITH COMBINATIONS OF 5-FU, LEUCOVORIN, AND COFACTOR

Materials and Methods

Mice

Balb/c mice were obtained from Charles River Laboratories. Mice were 6-8 weeks old at the start of all studies. Mice were maintained in isolated, hepa-filter ventilated cages with 4 mice per cage at LAB International's vivarium (San Diego, CA).

Drugs

5-Fluorouracil (5-FU) was obtained from Calbiochem. Folinic acid (leucovorin) and oxaliplatin were obtained from Sigma-Aldrich. CoFactor (5, 10 methylenetetrahydofolate) was manufactured by Eprova AG.

Balb/c Blood Analysis Study

Balb/c mice, 7 weeks old female mice, were injected for seven consecutive days with combinations of 5-FU, leucovorin, and CoFactor. All drugs were intraperitoneally injected (100microliters/mouse, 0.6mg/mouse/drug) using a 28 gauge insulin needle/syringe. 200-250microliters blood/mouse was collected by retro-orbital puncture into EDTA-coated microtainer tubes (VWR International) on days 0 (prior to drug injection), 8, and 13. Complete blood counts plus blood differentials were determined by Labcorp Corporation of America using a Bayer Advia 120 Hematology analyzer.

Results

In addition to its tumoricidal activity, 5-FU is cytotoxic towards normal cells, especially cells of the hematopoietic system due to its myelosuppressive effects. Because of the related chemical characteristics and modes of action of leucovorin and CoFactor,

we wanted to determine if there were similar toxicity profiles of 5-FU/CoFactor combination therapy. As such, we injected normal Balb/c mice with various combinations of 5-FU, leucovorin, and CoFactor (**Table 5**). Pretreatment, one week, and two weeks following treatment, we analyzed complete blood counts plus differentials for changes in blood parameters. Furthermore, we analyzed qualitative and quantitative measures of drug toxicity.

After one week of drug dosing, we observed all mice had drug-related toxicity including ruffled fur, moribundity, and dehydration. Within 12 days of initiation of drug treatment, all mice in the 5-FU only and 5-FU/leucovorin treatment groups had died. In contrast, 38% of mice (5 of 13) in the 5-FU/CoFactor treatment group were alive after 14 days. Kaplan-Meier survival curves were plotted for all treatment groups (**Figure 10**). Logrank statistical comparison of the 5-FU/CoFactor treatment group versus the 5-FU/Leucovorin treatment group indicated a significant difference in survival ($p < 0.05$).

Blood analysis also revealed differences in select blood cell types (**Figure 11**). We measured the following blood parameters: white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin content (MCHC), neutrophils, lymphocytes, platelets (PLT), eosinophils, basophils, and monocytes. One week following drug treatment, we observed significantly more white blood cells in 5-FU/CoFactor treated mice than 5-FU/leucovorin treated mice ($p < 0.05$, Student's t test). Among the white blood cell subsets, we observed significantly more platelets and neutrophils in the 5-FU/CoFactor treated group than the other treatment groups.

Since we observed differences in both platelet and neutrophil levels following 5-FU/CoFactor treatment, we assessed these cell types further. Using NCI grading criteria for toxicity, we calculated the percentage of mice with either combined grade 1/2 toxicity, grade 3 toxicity, or grade 4 toxicity. For platelets, we observed 25% of mice treated 5-FU alone developed grade 4 toxicity (**Figure 12**). In contrast, no grade 4 toxicity was noted for either 5-FU/leucovorin or 5-FU/CoFactor treated mice. However, unlike 5-FU/leucovorin mice with grade 3 toxicity (45%), only 15% of 5-FU/CoFactor treated mice developed grade 3 platelet toxicity. The remaining 5-FU/CoFactor treated

mice (85%) developing only grade 1 or 2 toxicity. As such, this data suggests 5-FU/CoFactor induces milder platelet toxicity than either 5-FU alone or 5-FU/leucovorin.

Similarly, we assessed the neutrophil toxicity profiles. In contrast to the platelet differences, the standard NCI grading system did not reveal noticeable neutrophil differences between treatment groups. For example, 100% of both 5-FU only and 5-FU/leucovorin treated mice developed grade 4 toxicity while 92% of 5-FU/CoFactor treated mice developed the same grade 4 toxicity. The remaining 8% of 5-FU/CoFactor treated mice developed grade 3 toxicity (**Figure 13**). However, closer analysis of mice that developed grade 4 toxicity revealed quantifiable neutrophil differences. We divided mice with grade 4 toxicity into subgroups based on their neutrophil cell count ranges following treatment (**Figure 14**). This analysis revealed that 100% of mice treated with 5-FU only, and 80% of 5-FU/leucovorin treated mice, had neutrophil cell counts between 0 and 99. In contrast, only 40% of 5-FU/CoFactor treated mice developed this lowest level neutrophil cell count. The majority of grade 4-rated 5-FU/CoFactor treated mice (50%) had neutrophil cell counts in the range of 200-499. Thus, this data suggests 5-FU/CoFactor results in milder neutrophil toxicity than either 5-FU alone or 5-FU/leucovorin.

EXAMPLE 4: WEIGHT LOSS TOXICITY ANALYSIS OF BALB/C MICE TREATED WITH COMBINATIONS OF 5-FU, LEUCOVORIN, COFACTOR, AND GEMCITABINE

Materials and Methods

Mice

Balb/c mice were obtained from Charles River Laboratories. Mice were 6-8 weeks old at the start of the study. Mice were maintained in isolated, hepa-filter ventilated cages with 4 mice per cage at LAB International's vivarium (San Diego, CA).

Drugs

5-Fluorouracil (5-FU) and folinic acid (leucovorin) were obtained from Sigma-Aldrich. CoFactor (5, 10 methylenetetrahydofolate) was manufactured by Eprova AG. Gemcitabine was manufactured by Eli Lilly and purchased from Myoderm Inc..

Balb/c Weight Analysis Study

Balb/c female mice were injected with combinations of 5-FU, leucovorin, CoFactor, and gemcitabine. 5-FU, leucovorin, and CoFactor were intraperitoneally injected (100microliters/mouse, 0.6mg/mouse/drug) for five consecutive days (days 1-5). Gemcitabine was intraperitoneally injected (100microliters/mouse, 100micrograms/mouse) every three days (days 1, 4, and 7). All drugs were injected using a 27 gauge insulin needle/syringe. Mouse weights were measured using an analytical balance prior to initiation of drug dosing (pretreatment) and on day 8.

Results

A known toxicity of 5-FU is gastrointestinal toxicity and associated weight loss. It is reported that leucovorin can potentially exacerbate gastrointestinal toxicity. Furthermore, gemcitabine, the current standard therapy for pancreatic cancer, has its own associated toxicity profile. While combination 5-FU/gemcitabine and 5-FU/leucovorin/gemcitabine therapy have been examined in the clinic and shown to have enhanced clinical activity, these combinations typically display more severe toxicity than gemcitabine alone or 5-FU/leucovorin alone. Because of the related chemical characteristics and modes of action of leucovorin and CoFactor, we wanted to investigate the toxicity profiles of 5-FU/CoFactor in combination with gemcitabine, since 5-FU/CoFactor/gemcitabine combination therapy is a potential treatment regimen for pancreatic cancer. Furthermore, we wanted to expand upon our previous toxicity analysis of combination 5-FU/CoFactor and determine if this combo has additional non-obvious toxicity profiles compared to either 5-FU/leucovorin or 5-FU alone. As such, we injected normal Balb/c mice with various combinations of 5-FU, leucovorin, CoFactor, and gemcitabine (**Table 6**). Pretreatment and one week following treatment initiation, we examined weight loss/gain as a measure of gastrointestinal toxicity.

Prior to initiation of drug administration (pre-treatment), randomized groups of mice (12 per group) displayed similar mean body weights. Following treatment (day 8), mouse weights decreased in all treatment groups. Using the National Cancer Institute's

(NCI) Common Terminology Criteria for Adverse Events, the severity of weight loss was plotted for each treatment group (**Figure 15**). Toxicity grading is based on the percentage weight loss from the starting baseline weight (**Table 7**). These results show 5-FU/CoFactor induced significantly less ($p < 0.05$, Fisher's exact test) grade 2-3 toxicity (50%) than either 5-FU alone or combination 5-FU/leucovorin treatment (100% grade 2-3 toxicity for both treatment groups).

While gemcitabine treatment alone did not induce weight loss toxicity greater than grade 1 due to administration of a subtoxic concentration, addition of gemcitabine to either 5-FU/leucovorin or 5-FU/CoFactor treatment resulted in 100% of mice with grade-3 toxicity (**Figure 15**). However, quantitative differences in the percentage weight loss could be detected between these treatment groups (**Figure 16**). This data suggests CoFactor protects mice from weight loss more effectively than leucovorin when used in combination with dual-cytotoxic drugs 5-FU and gemcitabine. While 92% of 5-FU/leucovorin/gemcitabine treated mice had >25% weight loss, significantly less ($p < 0.05$, Fisher's exact test) 5-FU/CoFactor/gemcitabine treated mice had this severity of weight loss (33% of mice).

Mouse survival was also followed over time for each treatment group (**Figure 17**). 5-FU/leucovorin and 5-FU/CoFactor groups both had significantly greater percentages ($p < 0.05$, Logrank test) of mice survive for up to 14 days (83% for each group), compared to mice treated with only 5-FU only (36%). The shortest survival time was observed in the triple drug combinations of either 5-FU/leucovorin/gemcitabine or 5-FU/CoFactor/gemcitabine in which 100% of the mice died prior to day 14. However, 5-FU/CoFactor/gemcitabine mice did survive significantly longer (9 days, $p < 0.05$, Logrank test) than 5-FU/leucovorin/gemcitabine treated mice (8 days). This correlates with the less severe weight loss toxicity described above for the 5-FU/CoFactor/gemcitabine combination group, and again suggests CoFactor induces milder weight loss compared to leucovorin when used with combination 5-FU/gemcitabine regimens.

EXAMPLE 2: LYMPHOCYTE ANALYSIS OF BALB/C MICE TREATED WITH COMBINATIONS OF 5-FU, LEUCOVORIN, AND COFACTOR

Materials and Methods

Mice

Balb/c mice were obtained from Charles River Laboratories. Mice were 6-8 weeks old at the start of all studies. Mice were maintained in isolated, hepa-filter ventilated cages with 4 mice per cage at LAB International's vivarium (San Diego, CA).

Drugs

5-Fluorouracil (5-FU) was obtained from Calbiochem. Folinic acid (leucovorin) and oxaliplatin were obtained from Sigma-Aldrich. CoFactor (5, 10 methylenetetrahydofolate) was manufactured by Eprova AG.

Balb/c Blood Analysis Study

Balb/c mice, 7 weeks old female mice, were injected for seven consecutive days with combinations of 5-FU, leucovorin, and CoFactor. All drugs were intraperitoneally injected (100microliters/mouse, 0.6mg/mouse/drug) using a 28 gauge insulin needle/syringe. 200-250microliters blood/mouse was collected by retro-orbital puncture into EDTA-coated microtainer tubes (VWR International) on days 0 (prior to drug injection), 8, and 13. Complete blood counts plus blood differentials were determined by Labcorp Corporation of America using a Bayer Advia 120 Hematology analyzer.

Results

Additional analysis of the previously described experiment in the original provisional patent filing (Example 3 of original provisional) has revealed further toxicity differences between treatments groups. As originally described, we noted protection in white blood cells, including platelets and neutrophils, in the 5-FU/CoFactor treatment group compared to 5-FU/leucovorin and 5-FU alone. New analysis of the data, using NCI toxicity grading based on the percentage of baseline lymphocyte levels (**Table 18**), also shows greater protection of lymphocytes in the 5-FU/CoFactor treatment group compared to the other groups (**Figure 18**). While 100% of mice in the 5-FU only and 5-FU/leucovorin treatment groups developed Grade 3-4 lymphopenia, significantly less ($p < 0.05$, Fisher's exact test) mice in the 5-FU/CoFactor treatment group developed this

level of toxicity (62%). As such, this data suggests 5-FU/CoFactor induces milder lymphocyte toxicity than either 5-FU alone or 5-FU/leucovorin.

Antitumor activity of combination 5,10-methylenetetrahydrofolate, 5-fluorouracil, and anti-vascular endothelial growth factor against human colorectal HT-29 tumors in nude mice.

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Background: Folinic acid (leucovorin) has been used as the standard combination therapy as a modulator of 5-fluorouracil (5-FU) for cancer treatment. However, leucovorin is inactive directly and must undergo several metabolic transformations to its active metabolite 5,10-methylenetetrahydrofolate (CoFactor) to be effective. In contrast, CoFactor supplies 5,10-methylenetetrahydrofolate directly and has demonstrated enhancement of the antitumor effects of 5-FU in Phase I/II human clinical trials for colorectal and breast cancer. To determine if the antitumor activity of CoFactor/5-FU could be enhanced further, we examined its use in combination with a recombinant antibody specific for vascular endothelial growth factor (aVEGF), an inhibitor of angiogenesis, against human colorectal HT-29 tumors in nude mice. Methods: 6-8 week old nude mice (nu/nu) were inoculated subcutaneously with 2 x 10^6 HT-29 cells. When tumors reached 0.1 to 0.3 cm^3 in volume, mice were treated with various combinations of 5-FU, CoFactor, leucovorin, and aVEGF administered by intraperitoneal injection. All drugs were dosed daily (0.6 mg/mouse/drug) for five consecutive days with the exception of aVEGF, dosed once (100 mg/mouse) on day 1. In addition, CoFactor or leucovorin were injected 20 minutes prior to 5-FU injection. Tumor volumes were calculated every 2 to 3 days. Results: One month following treatment, we observed smaller mean tumor volumes in mice treated with combination CoFactor/aVEGF/5-FU (0.48 cm^3 ± 0.1, n=8, mean ± SEM) than mice treated with either 5-FU alone (0.75 cm^3 ± 0.1), CoFactor/FU (0.52 cm^3 ± 0.08), or leucovorin/5-FU (0.71 cm^3 ± 0.09). Furthermore, there was greater survival of mice treated with CoFactor/5-FU either with or without aVEGF (57% and 88%, respectively) compared to mice treated with only 5-FU (25%). Conclusions: This study suggests combination CoFactor/aVEGF/5-FU treatment might have utility as a colorectal tumor therapy with greater antitumor activity than standard 5-FU therapies.

Bibliography

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Plasencia, Taron, Martinez, McLeod, Rosell, and Abad (2002) Molecular aspects involved in chemotherapy response in sensitive and 5FU resistant colorectal cancer (CRC) cell lines. American Society for Clinical Oncology Annual Meeting Abstract No. 2188.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

All references cited herein, including those in the bibliography, are incorporated by reference in their entireties.

Table 1. Investigational Colorectal Drugs

Category	Drug	Company	Mechanism
1	ABT-751	Abbott Laboratories	Microtubulin inhibitor
1	Epothilone D	Kosan Biosciences	Microtubulin Inhibitor
2	105AD7	Onyvax	Anti-idiotype vaccine
2	BCG	Intracel	Mycobacterium Autologous Vaccine
2	EP2101	Epimmune	Peptide Vaccine
2	Mutant ras + IL-2 vaccine	NCI	Dendritic vaccine
2	SGN-00101	Stressgen	BCG vaccine
3	ABX-EGF (panitumumab)	Abgenix	Anti-EGFR
3	GW572016	GlaxoSmithKline	EGFR/ERBb2 inhibitor
3	BAY 43-9006	Bayer/Onyx	RAF/VEGF signal inhibitor
4	EKB-569	Wyeth-Ayerst	EGF Receptor kinase inhibitor
4	Erlotinib	Genentech	Tyrosine kinase inhibitor
4	Gefitinab (Iressa)	AstraZeneca	EGFR tyrosine kinase inhibitor
4	PTK787/ZK 222584	Novartis	VEGFR Tyrosine Kinase Inhibitor
4	E7070	Eisai Medical Research	Cdk2 and cyclin E inhibitor
5	Celecoxib (Celebrex)	Pfizer	Nonsteroidal Anti-inflammatory
5	Rofecoxib (Vioxx)	Merck	Nonsteroidal Anti-inflammatory
6	GM-CSF		Cytokine
6	Interferon alpha		Cytokine
6	Interferon beta		Cytokine
6	TNFerade	Genvec	Adenovirus TNF Cytokine
7	DAVANAT	Pro-Pharmaceuticals	Carbohydrate binder that targets 5-FU to cell
7	Etoposide	Schering Plough	Farnesyl transferase inhibitor
7	LMB-9	NCI	Lewis Y antibody
8	Imatinib (Gleevec)	Novartis	
8	Oblimersin	Genta	BCL-2 inhibitor
9	Tezacitabine	Chiron	Nucleoside Analogue
10	Antineoplaston	Burzynski Research Inst.	
10	Mistletoe extract (Helixor A)	NCCAM	
10	N-phosphonacetyl-L-aspartic acid (PALA)		5-FU modulator
10	PHY906	PhytoCeutica	Anti-diarrhea
10	Talaporfin sodium (LS11)	Light Sciences Corp.	Light activated drug
10	Thalidomide	NCI	Anti-vascular

[1]Microtubulin Inhibitor
[2]Vaccine
[3]EGFR/VEGFR Target
[4]Tyrosine Kinase/Transcription Factor Inhibitor
[5]Nonsteroidal Anti-Inflammatory
[6]Cytokine
[7]Carbohydrate/Lipid
[8]Apoptosis Regulator
[9]Nucleoside Analogue
[10]Miscellaneous

Table 2. Mouse Treatment Groups

Group #	*Treatment*	*Mice/group*
1	Saline	8
2	5-FU	8
3	CoFactor	8
4	Anti-VEGF	8
5	Oxaliplatin	8
6	5-FU/Leucovorin	8
7	5-FU/CoFactor	8
8	5-FU/anti-VEGF	8
9	5-FU/Oxaliplatin	8
10	5-FU/CoFactor/anti-VEGF	8
11	5-FU/CoFactor/Oxaliplatin	8
Total		**88**

Table 3. Mouse Treatment Groups

Group #	*Treatment*	*Mice/group*
1	Saline	12
2	5-FU	12
3	5-FU/Leucovorin	12
4	5-FU/CoFactor	12
5	5-FU/Avastin	12
6	5-FU/Leucovorin/Avastin	12
7	5-FU/CoFactor/Avastin	12
Total		**84**

Table 4. Tumor Doubling Times

Group #	*Treatment*	*Doubling Time (days)*
1	Saline	7.6
2	5-FU	7.4
3	5-FU/Leucovorin	8.5
4	5-FU/CoFactor	8.2
5	5-FU/Avastin	8.4
6	5-FU/Leucovorin/Avastin	8.6
7	5-FU/CoFactor/Avastin	9.9

Table 5. Balb/c Mouse Treatment Groups

Group #	*Treatment*	*Mice/group*
1	5-FU	12
2	5-FU/Leucovorin	13
3	5-FU/CoFactor	13
Total		**38**

Table 6. Balb/c Mouse Treatment Groups

Group #	*Treatment*	*Mice/group*
1	5-FU	11
2	5-FU/Leucovorin	12
3	5-FU/CoFactor	12
4	Gemcitabine	12
5	5-FU/Leucovorin/Gemcitabine	12
6	5-FU/CoFactor/Gemcitabine	12
Total		**71**

Table 7. National Cancer Institute Weight Loss Toxicity Grades

Toxicity	*Grade 0*	*Grade 1*	*Grade 2*	*Grade 3*
Weight Loss	<5%	5-<10%	10-<20%	≥20%

Table 8. National Cancer Institute Lymphopenia Toxicity Grades

Toxicity	*Grade 1*	*Grade 2*	*Grade 3*	*Grade 4*
Lymphopenia	75-<100%LLN	50-<75%LLN	25-<50%LLN	<25%LLN

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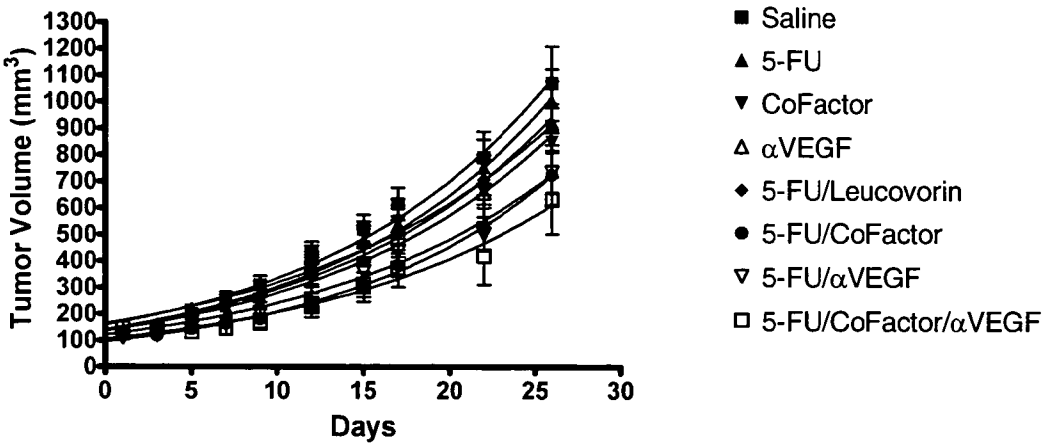


Figure 1. HT-29 Tumor Growth Kinetics. HT-29 tumor volumes were plotted against time from treatment initiation. Mean tumor volume ± standard error of the mean are plotted. Curves were generated by best-fit analysis.

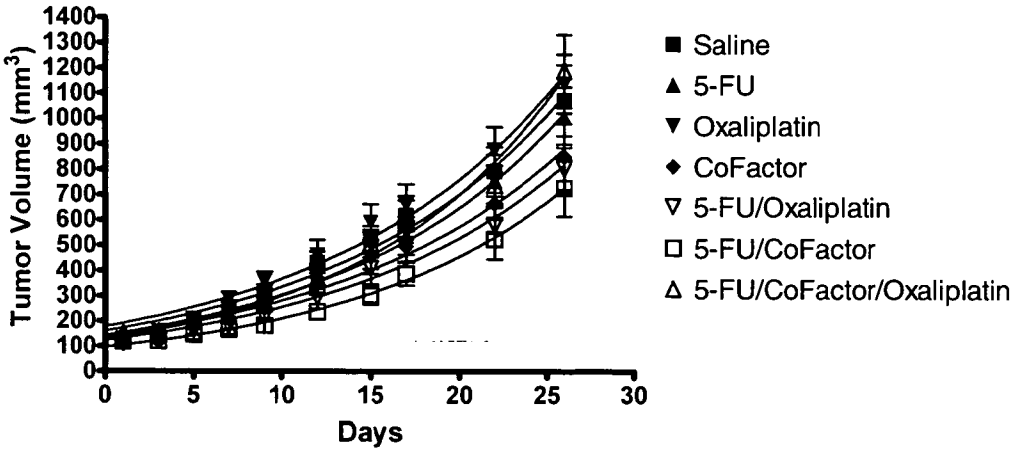


Figure 2. HT-29 Tumor Growth Kinetics. HT-29 tumor volumes were plotted against time from treatment initiation. Mean tumor volume ± standard error of the mean are plotted. Curves were generated by best-fit analysis.

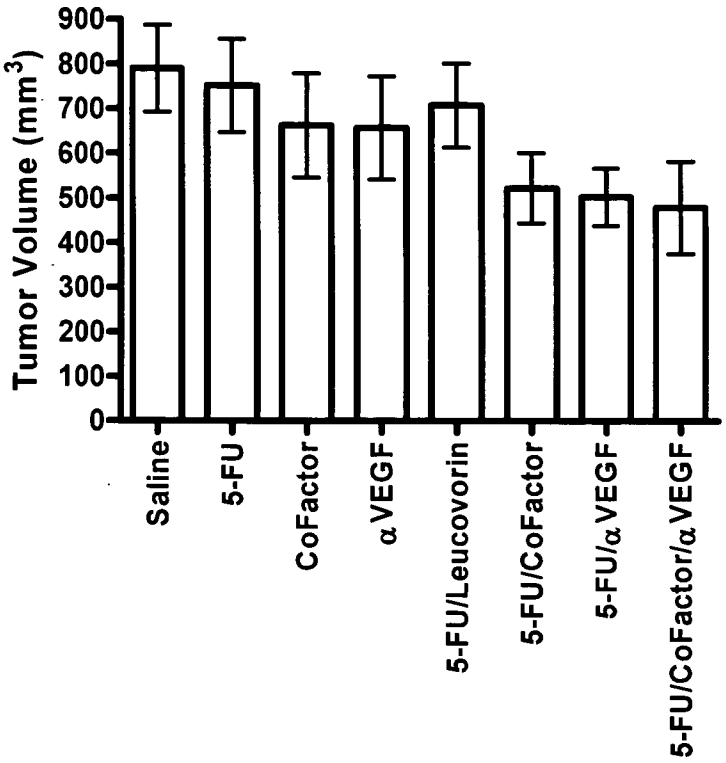


Figure 3. Mean Tumor Volumes Following Treatment. Mean tumor volumes 22 days following treatment initiation were plotted for each treatment group. Error bars represent standard error of the means.

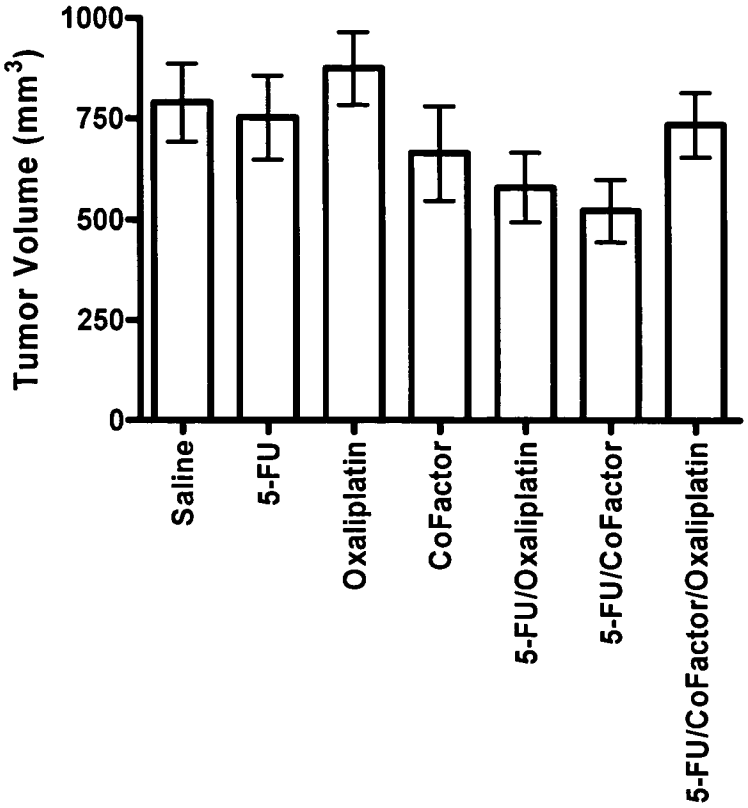


Figure 4. Mean Tumor Volumes Following Treatment. Mean tumor volumes 22 days following treatment initiation were plotted for each treatment group. Error bars represent standard error of the means.

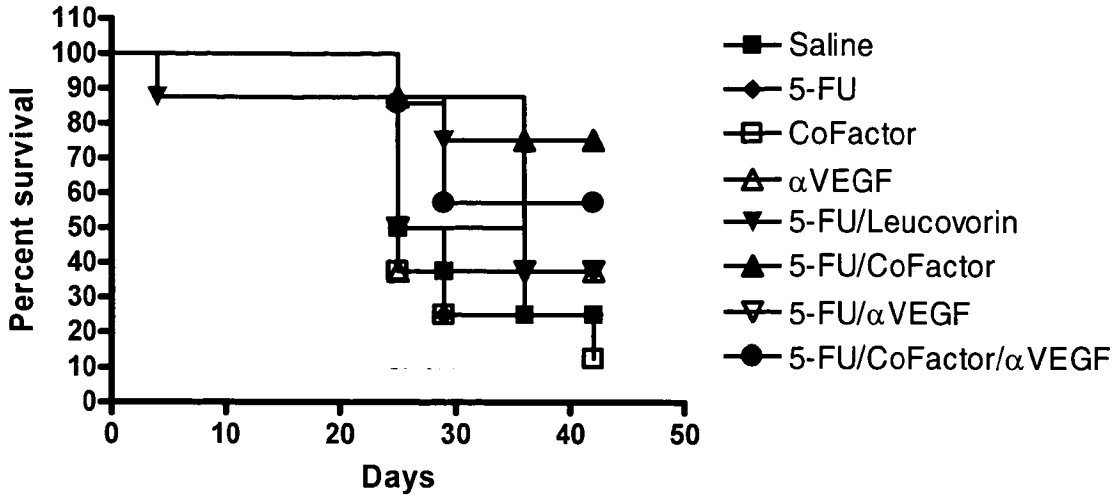


Figure 5. Nude Mice Survival Curves. Kaplan-Meier plot of survival of Nude mice following treatment with combination of 5-FU, CoFactor, leucovorin, and anti-VEGF.

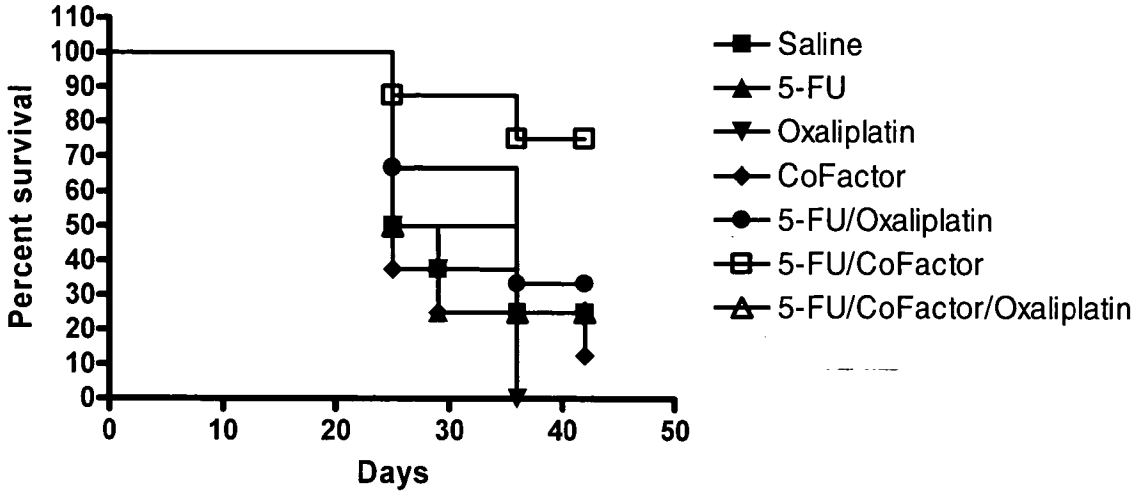


Figure 6. Nude Mice Survival Curves. Kaplan-Meier plot of survival of Nude mice following treatment with combination of 5-FU, CoFactor, and oxaliplatin.

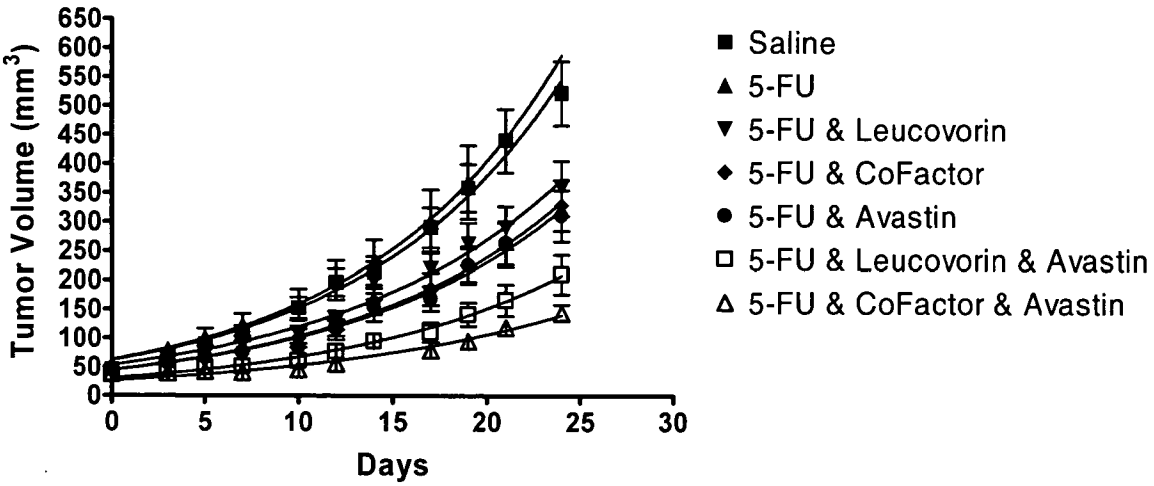


Figure 7. HT-29 Tumor Growth Kinetics. HT-29 tumor volumes were plotted against time from treatment initiation. Mean tumor volume ± standard error of the mean are plotted. Curves were generated by best-fit analysis.

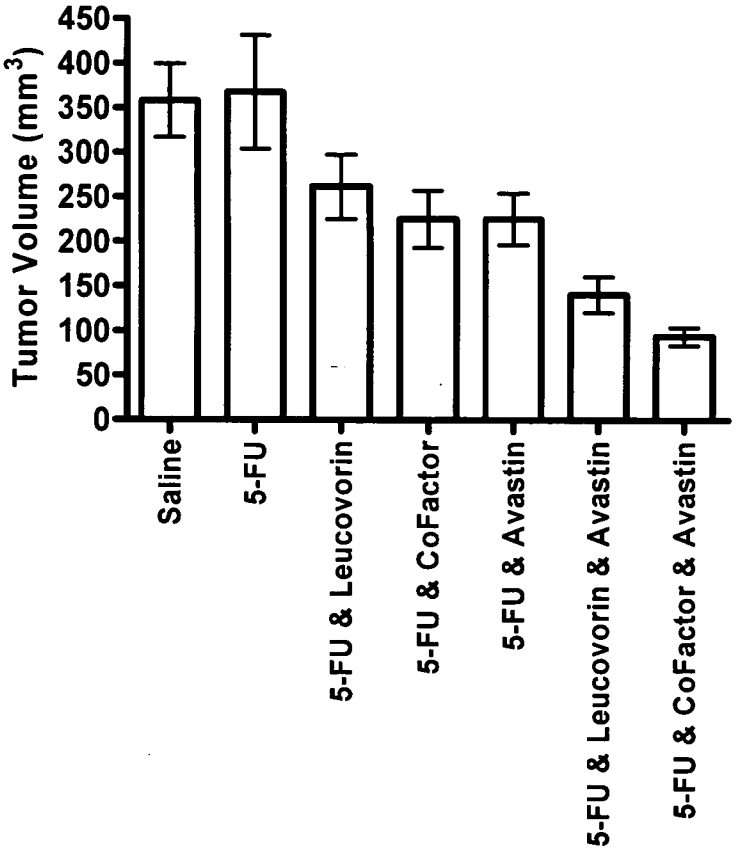


Figure 8. Mean Tumor Volumes Following Treatment. Mean tumor volumes 19 days following treatment initiation were plotted for each treatment group. Error bars represent standard error of the means.

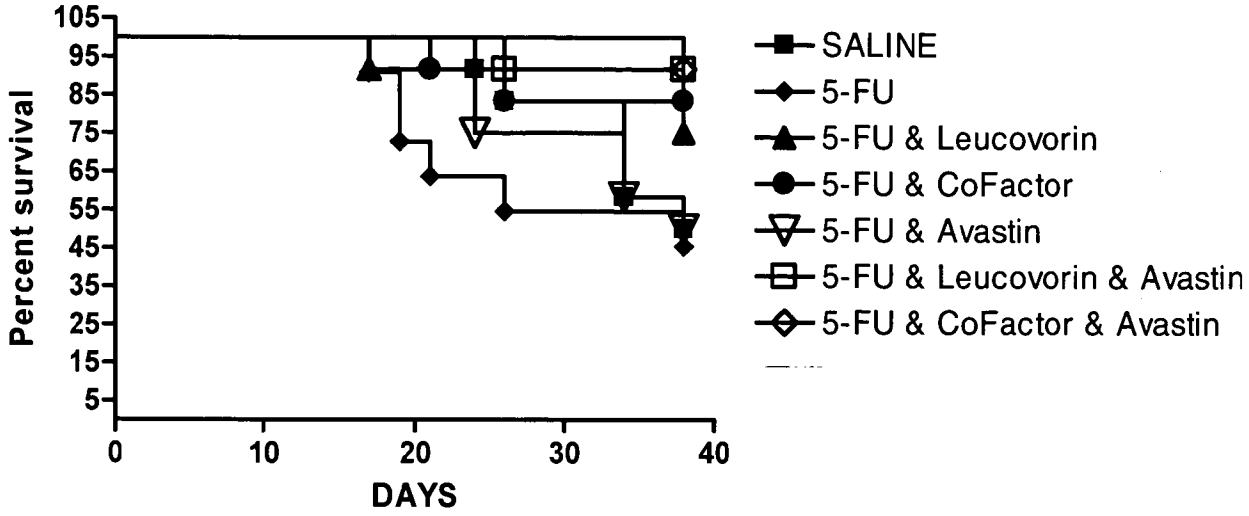


Figure 9. Nude Mice Survival Curves. Kaplan-Meier plot of survival of Nude mice following treatment with combination of 5-FU, CoFactor, and Avastin.

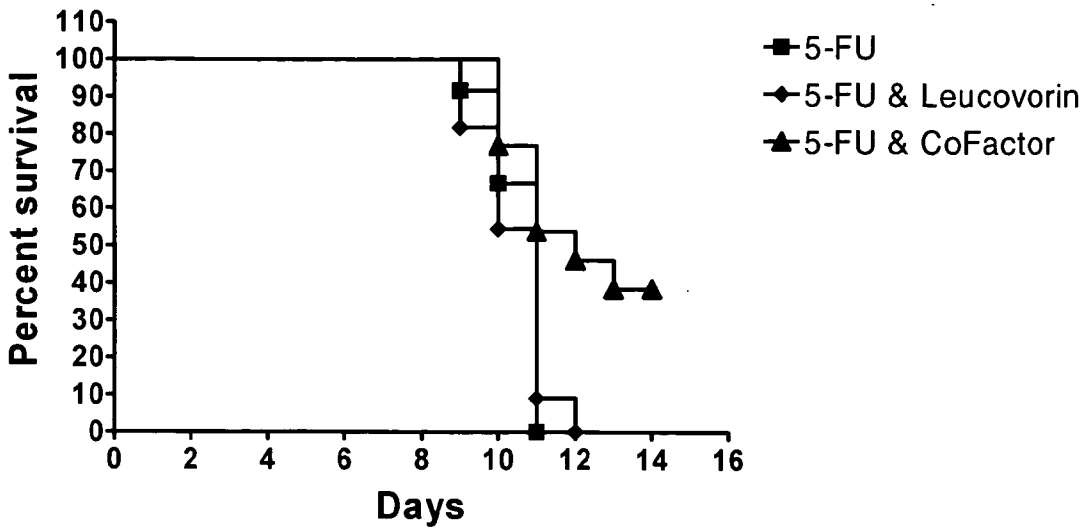


Figure 10. Balb/c Survival Curves. Kaplan-Meier plot of survival of Balb/c mice following 5-FU, 5-FU/leucovorin, and 5-FU/CoFactor treatment.

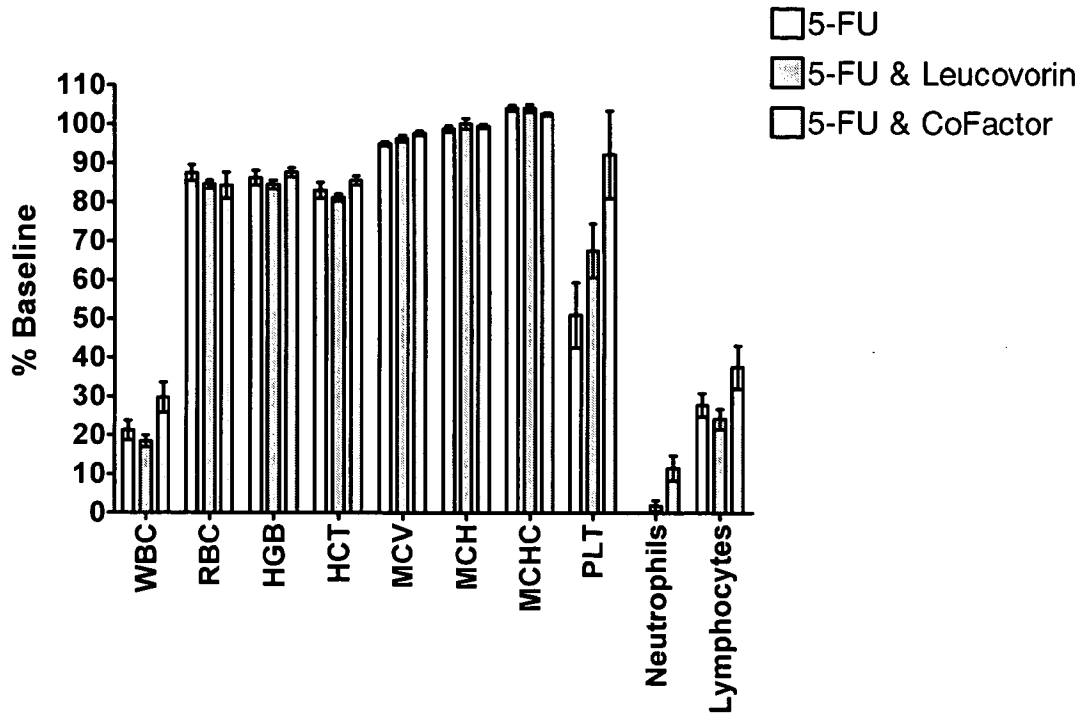


Figure 11. Balb/c Blood Analysis. Blood measurements taken 1 week after drug therapy were divided by the pre-treatment blood measurements to calculate the percentage baseline measurement plotted in the graph. Mean data values ± standard errors of the means are plotted for each treatment group.

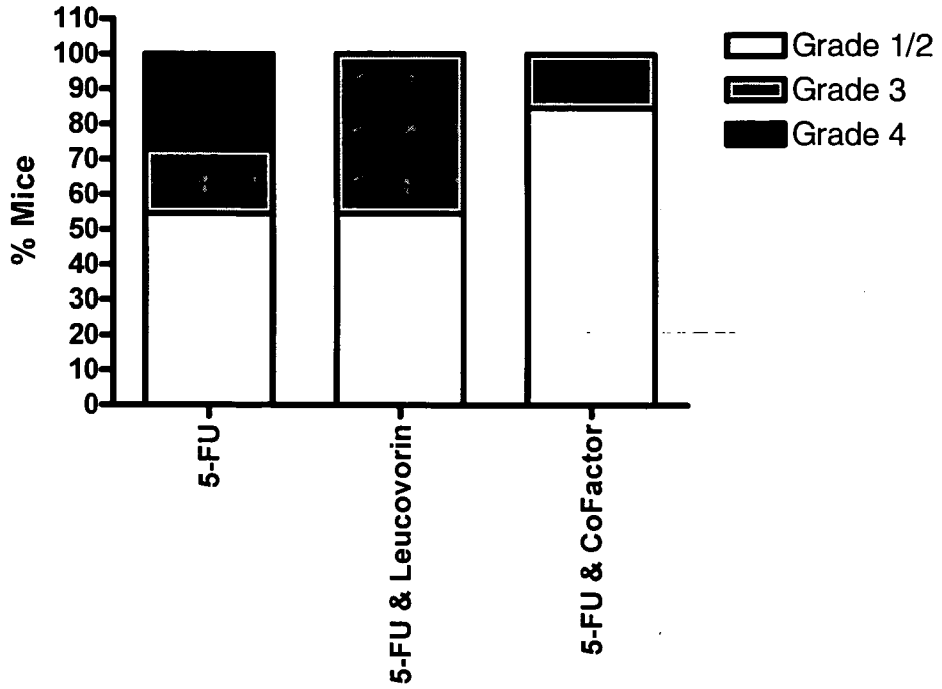


Figure 12. Platelet Toxicity Grading. One week following drug treatment, the grade of platelet toxicity was calculated for each mouse. The percentage of mice with grade 1 or 2, grade 3, and grade 4 toxicity are plotted.

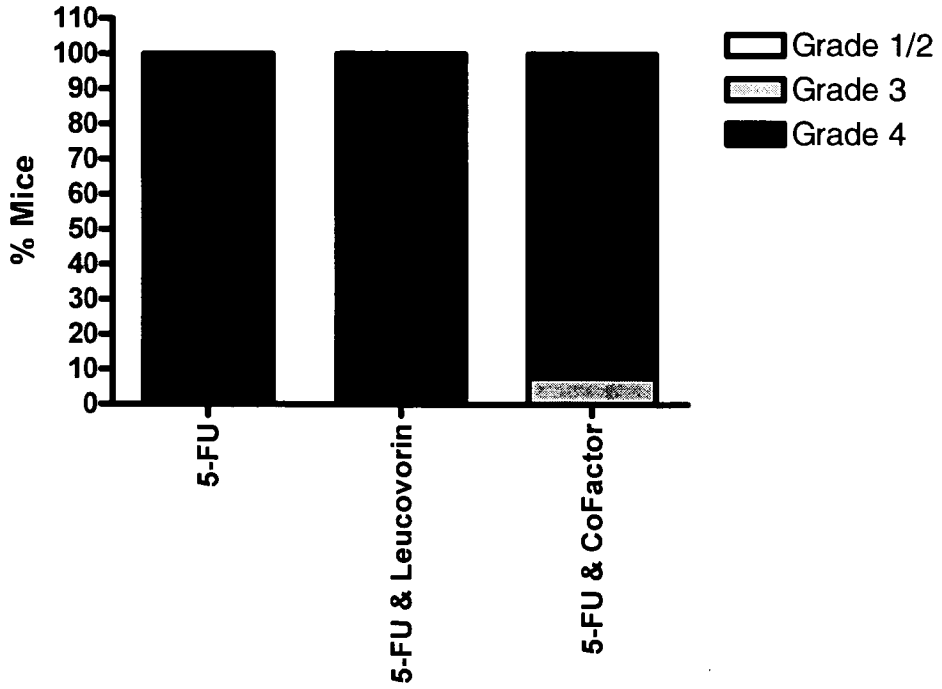


Figure 13. Neutrophil Toxicity Grading. One week following drug treatment, the grade of neutrophil toxicity was calculated for each mouse. The percentage of mice with grade 1 or 2, grade 3, and grade 4 toxicity are plotted.

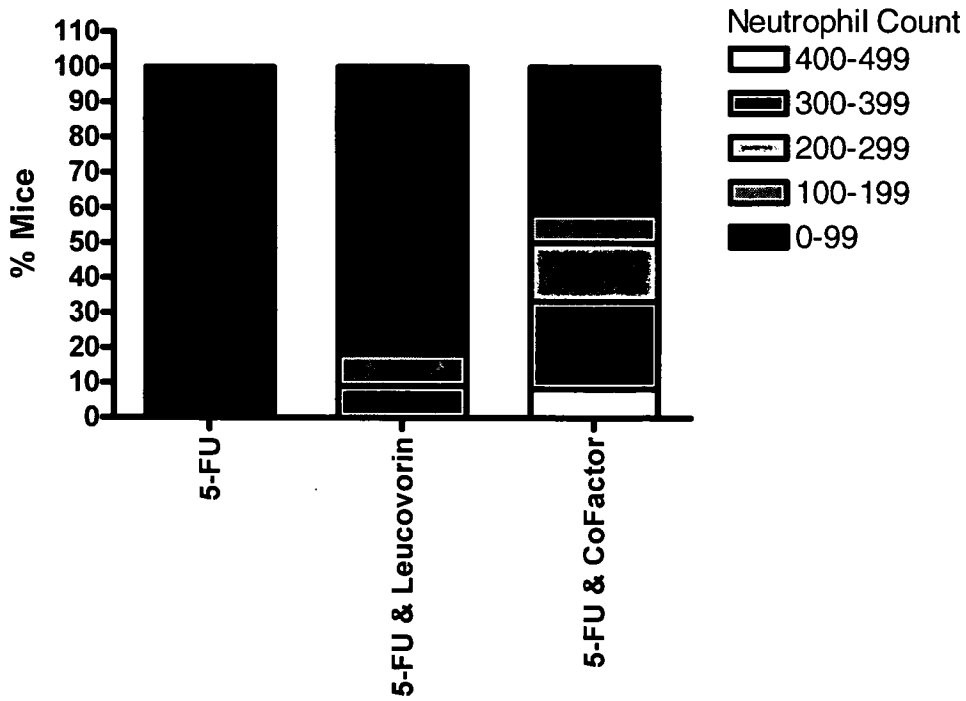


Figure 14. Grade 4 Neutrophil Toxicity Analysis. One week following drug treatment, mice with grade 4 neutrophil toxicity were subdivided based on their absolute neutrophil counts. The percentage of these mice with the legend-indicated neutrophil cell counts is plotted.

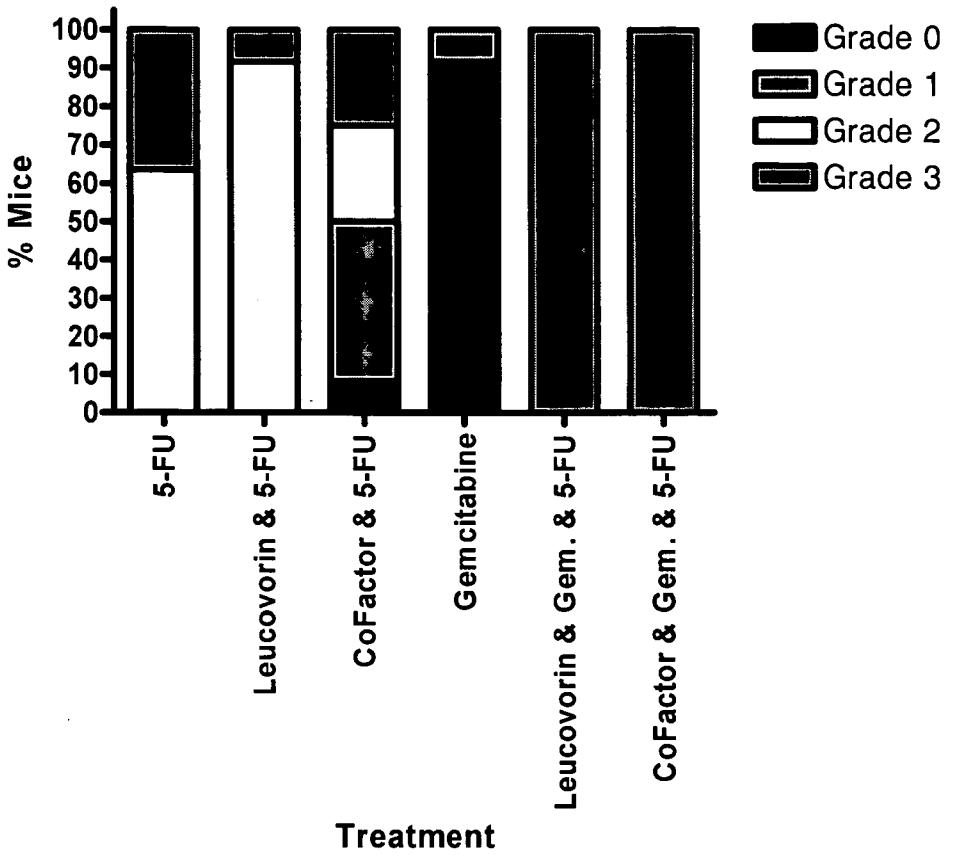


Figure 15. Weight Loss Toxicity Grading. One week following drug treatment, the grade of weight loss toxicity was calculated for each mouse. The percentage of mice with grade 0, 1, 2, and 3 toxicity are plotted. Gem = Gemcitabine

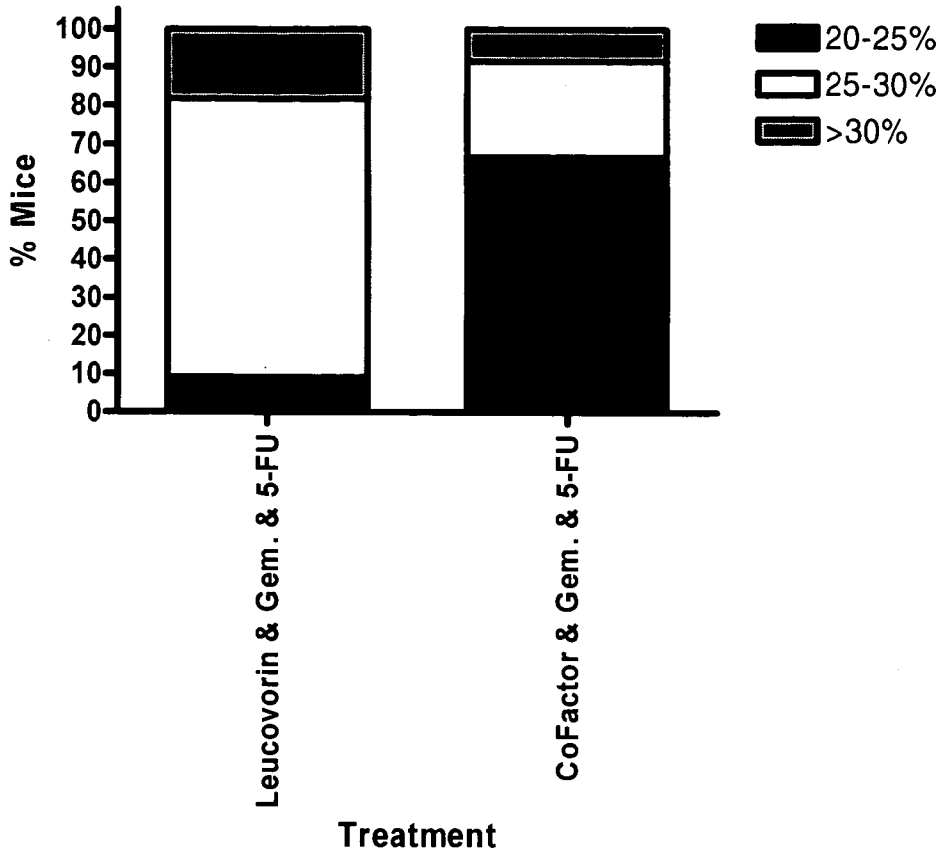


Figure 16. Percent Weight Loss of Gemcitabine Containing Treatment Groups. One week following drug treatment, the percentage weight loss from the starting baseline weights were calculated for each mouse. The percentage of mice that fell with the ranges of weight loss indicated in the legend was then plotted. Gem = Gemcitabine

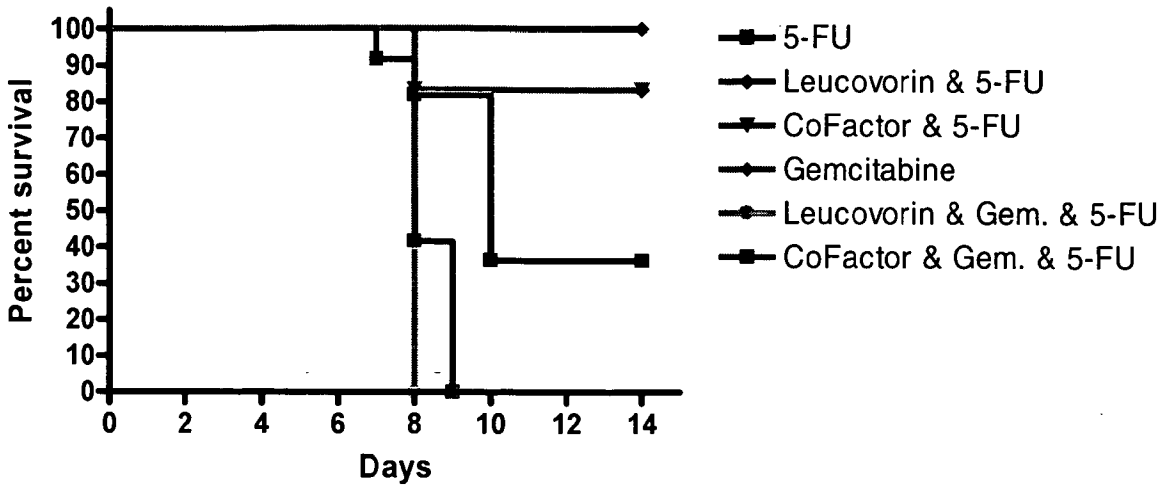


Figure 17. Balb/c Survival Curves. Kaplan-Meier plot of survival of Balb/c mice following treatment. Gem = Gemcitabine

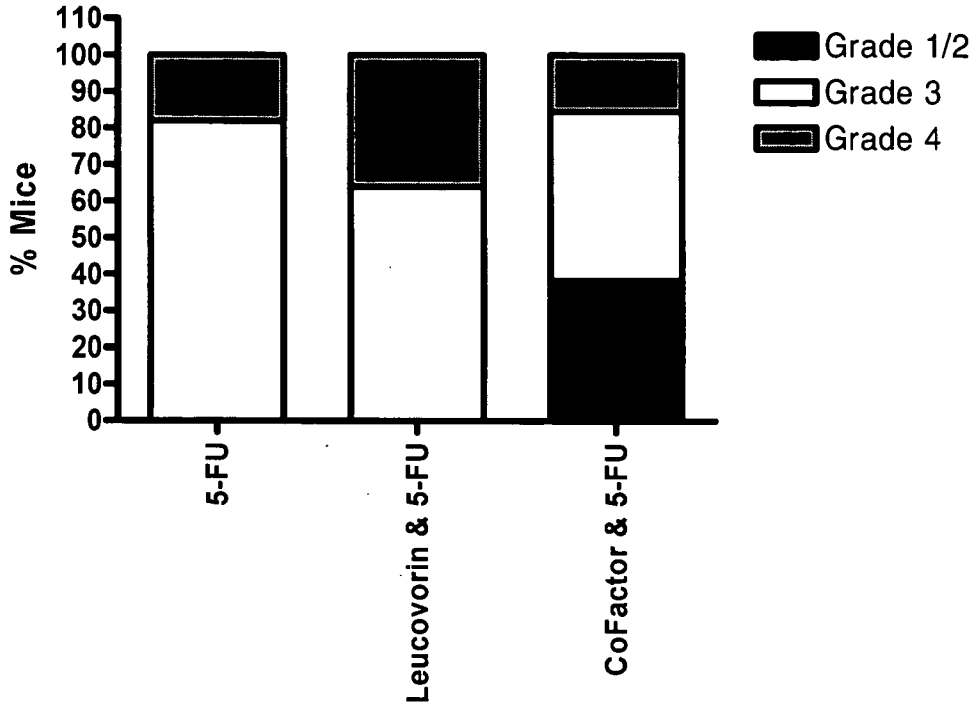


Figure 18. Lymphopenia Toxicity Grading. One week following drug treatment, the grade of lymphopenia was calculated for each mouse. The percentage of mice with grade 1/2, grade 3, and grade 4 toxicity are plotted.

5

CoFactor Evidence for Reduced Toxicities:

Reduced gastrointestinal toxicity using CoFactor

- **CF/5FU induced significantly less grade 2-3 toxicity (50%) than other treatment groups**

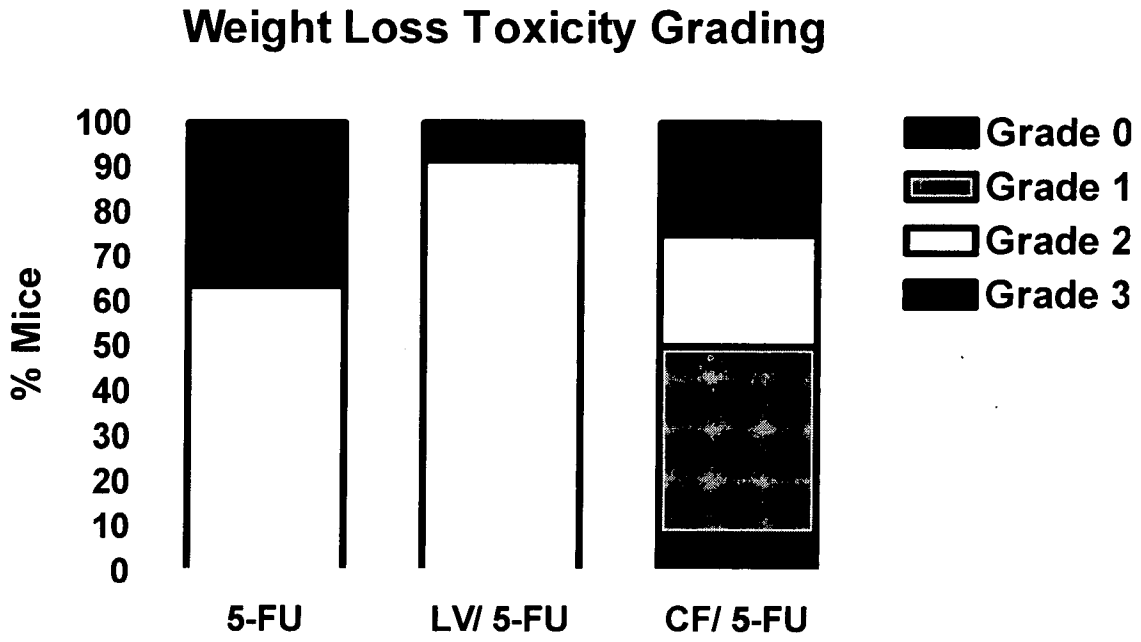


Figure 19

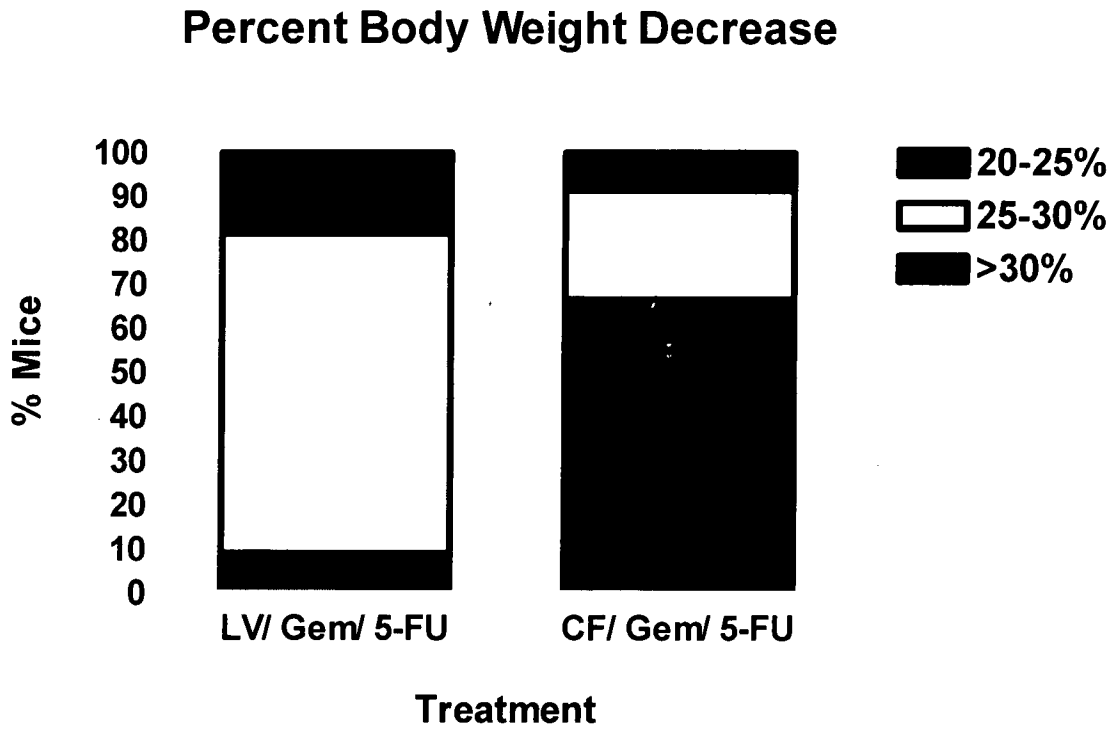
BALB/c, n=12 per group

Mark J. Cantwell, Joan M. Robbins, data not published, 2004. Mouse were weighed on Day 8 post-treatment. Grade 0, 1, 2, 3 defined as weight loss of <5%, 5-10%, 10-20% and ≥ 20%, respectively.

CoFactor Evidence for Reduced Toxicities:

Reduced gastrointestinal toxicity using CoFactor

- **92% of LV/G/5FU treated mice had >25% wt loss**
- **33% of CF/G/5FU treated mice had >25% wt loss**



BALB/c, n=12 per group

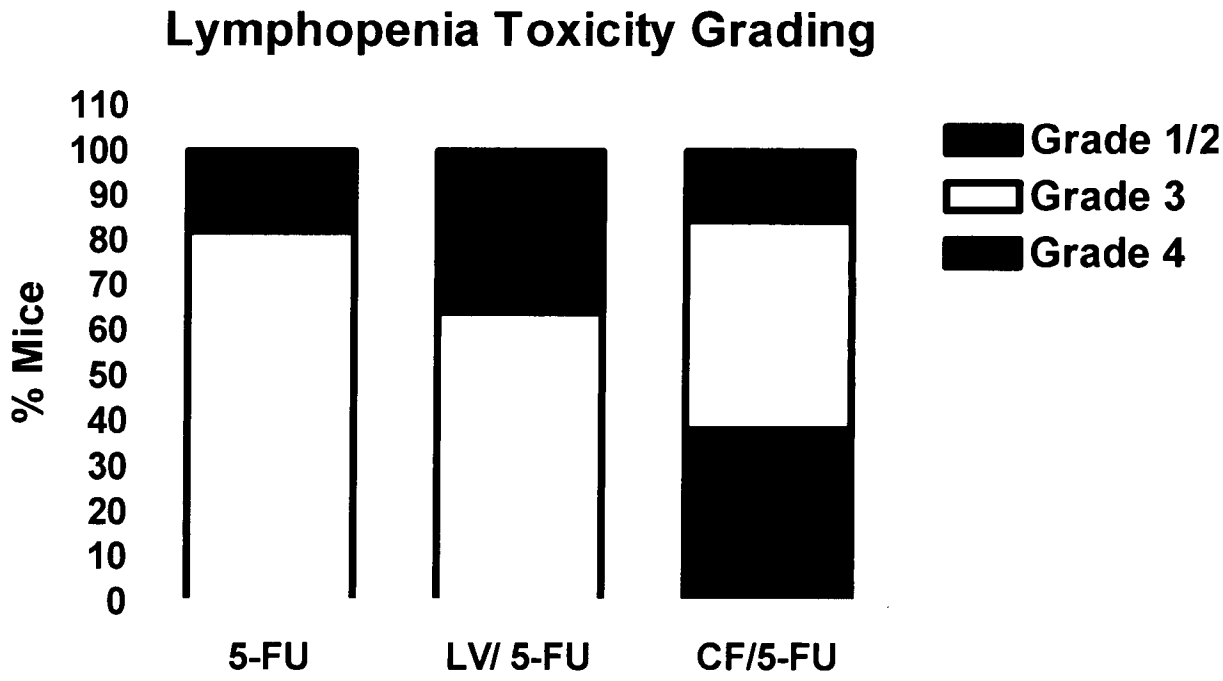
Figure 20

Mark J. Cantwell, Joan M. Robbins, data not published, 2004. Mouse were weighed on Day 8 post-treatment

CoFactor Evidence for Reduced Toxicities:

Reduced Lymphopenia using CoFactor

- **CF induces milder lymphocyte toxicity**
- **Greater lympho-cyte protection in the CF/5-FU treatment group compared to the other groups**



BALB/c, n=12 per group

Figure 21

Mark J. Cantwell, Joan M. Robbins, data not published, 2004.